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13. ABSTRACT (Maximum 200 Words)

TRE2-HA-FIP200-IRES2-EGFP-hβg3' DNA fragment was used to create transgenic mice by pronuclear injection into fertilized FVB/N eggs. Three independent TRE2-FIP200 founders were identified with PCR screening with EGFP primers. All three founders and their transgenic offspring are clinically normal, fertile, their litters are of normal size, their pups exhibit normal growth-rate. Mammary gland morphology is normal in all physiological stages in the TRE2-FIP200 mice when compared to wild-type littermates. Double transgenic offspring were created by mating TRE2-FIP200 transgenic mice to the regulator (MMTV-rtTA) mice. The double transgeninc mice are clinically normal, fertile, their litters are of normal size, their pups exhibit normal growth-rate. After doxycycline supplementation of the drinking water the transgenic FIP200 message can be detected with RT-PCR in one line (c3) of double transgenic animals. So far, attempts to detect transgenic FIP200 protein with immunoblotting, immunoprecipitation, and immunohistochemistry were unsuccessful. Experiments are planned to solve this problem. Experiments are being conducted to monitor mammary gland development in the double transgenic animals during pregnancy with or without doxycycline treatment.

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Bulleted list of key accomplishments

- **Three independent TRE2-HA-FIP200-IRES2-EGFP-hβg3' transgenic lines were created (b1, b7, and c3)**
- **All TRE2-FIP200 transgenic lines are phenotypically normal and fertile**
- **Mating between TRE2-FIP200 and MMTV-rtTA lines was successful and the double transgenic offspring are all phenotypically normal and fertile**
- **Double transgenic animals were exposed to doxycycline and transgene expression in the animals of the c3 line was observed with RT-PCR**
- **Both the TRE2-FIP200 and the MMTV-rtTA colonies were expanded to accommodate future needs**
- **Double transgenic animals were created and are being exposed to doxycycline to study mammary gland morphology during postnatal development and pregnancy**

Predoctoral Traineeship Award
Department of Defense Breast Cancer Research Program
Annual Report 2005

Research Accomplishments

Investigating the role of FIP200 in mammary carcinogenesis using a transgenic mouse model

Aim 1. **Generation of transgenic mice, examination of FIP200 transgene expression and the phenotype of the transgenic animals**

a. Genotypic screening of the TRE2-FIP200 transgenic mice

Gel-purified 11.9-kb TRE2-HA-FIP200-IRES2-EGFP-hβg3' DNA fragment at a concentration of 3 μg/ml was microinjected into the pronuclei of fertilized mouse eggs of FVB/N strain purchased from the Jackson Laboratory (Bar Harbor, ME). After culturing the manipulated embryos *in vitro* to the two-cell stage, the embryos were then transferred into the oviducts of 0.5-d pseudopregnant female FVB mice (1). DNA was isolated from tail samples of the newborn pups using standard protocols (2). PCR reaction was employed to detect the presence of the transgene using EGFP primers (GFP03, 5'-acggcaagctgaccctgaagt-3', and GFP04, 5'-gcttctcgttggggtctttgc-3'). The PCR reaction consisted of the following steps: denaturation at 94°C for 3 min and 35 cycles of amplification (94°C for 1 min, 60°C for 1 min 20 s, and 72°C for 2 min), followed by a 2-min extension at 72°C (3).

Following pronuclear injection of the TRE2-HA-FIP200-IRES2-EGFP-hβg3' transgene 31 mice were born. Three animals (2 males: b7 and c3 and 1 female: b1) were found carrying the injected transgene. These founder animals were phenotypically normal and fertile. The founders were mated to wild-type FVB mice and their offspring were analyzed for the transmission of the TRE2-HA-FIP200-IRES2-EGFP-hβg3' transgene. Interestingly none of the first litters from these mating pairs had any transgenic offspring. However, transgenic offspring appeared either in the second litter (for b1 and c3) or in a later litter (for b7).

b. Macroscopic and microscopic phenotypic analysis of the TRE2-FIP200 transgenic mice

The F1 TRE2-FIP200 transgenic offspring were mated to littermate wild-type animals and to littermate transgenic animals to create heterozygous and homozygous TRE2-FIP200 transgenic animals, respectively. Both the homozygous and the heterozygous TRE2-FIP200 transgenic animals were phenotypically normal and fertile.

Breeding of transgenic animals started between 7-8 weeks of age and continued until 6 months of age. Some females that were euthanized after the breeding period were subjected to a complete necropsy, including preparing the right inguinal mammary gland for whole mounting to assess the development of the mammary gland.

The litters of the TRE2-FIP200 dams were of normal size for FVB mice: the first litters being smaller (6-8 pups) than the later litters (10-14 pups). All TRE2-FIP200 transgenic mice were of normal size and weight at birth and exhibited normal rate of growth during the suckling period. All TRE2-FIP200 dams nursed their pups adequately: milk in the stomach was visible through the abdominal wall during the immediate post-partum perion.

All adult TRE2-FIP200 mice were clinically normal; the morbidity in the colony was less than 1% affecting both TRE2-FIP200 transgenic and wild-type animals to the same extent. These illnesses were occasional cases of dermatitis (in both males and females) and rare cases of preputial gland abscesses. A few males (less then 1%) exhibited aggression towards other mice. Mice with dermatitis or preputial gland abscesses were appropriately treated by Lab Animal Service and were

euthanized if the condition did not improve. Mice with aggression were euthanized promptly after exhibiting these signs.

The commonly observed background lesions, such as lung adenoma (4) or persistent mammary gland hyperplasia (5) were not found in our colony, most likely because in our study mice were euthanized before they reached old age (12-24 months).

In addition to mice with clinical signs, at least five pairs of clinically healthy mice from each line (one TRE2-FIP200 transgenic and one wild-type) from the same litter were sacrificed to examine their major organs and the mammary gland histologically. The right inguinal mammary gland from each of these animals was also processed for whole mounting and the left inguinal gland was fixed in 4% paraformaldehyde for paraffin embedding. These animals were in different physiological stages (immature, less than 4 weeks of age; mature 6-8 weeks of age; pregnant, 14 days post coitus; lactating, 7 days post partum) to examine if there is any difference in these animals histologically, especially with regards to their mammary gland morphology. For routine histological examination the organ samples were fixed in cold, PBS-buffered fresh 4% paraformaldehyde at +4 °C for 16 hours. Then the tissues were washed in PBS three times for 20 minutes at room temperature and once in 65% ethyl-alcohol, then stored in 70% ethyl-alcohol until they were processed for paraffin embedding. The mammary glands for whole mounts were spread on glass slides and were fixed in Carnoy's fixative (100% ethyl alcohol:chloroform:glacial acetic acid, 6:3:1) for 4 hours at room temperature and stained with Carmine Alum Stain (0.2% carmine and 0.5% aluminum potassium sulfate) overnight at room temperature. Mammary gland whole mounts were examined under a dissecting microscope, photographed and the images of corresponding samples were compared. To compare images the frequency of branching of the mammary ducts and the appearance and/or size of the mammary end buds were judged subjectively. No difference was found in the mammary gland whole mount morphology between TRE2-FIP200 transgenic (all three lines) and wild-type animals in any physiological state. Histological examination mainly focused on the morphology of the mammary epithelial cells and on the epithelial adipose tissue ratio. In pregnant and lactating glands the size of the acini and the presence of lipid vacuoles in the acinar cells were also compared.

c. Mating transgenic lines (TRE2-FIP200 and MMTV-rtTA) to create double transgenic offspring

Three mice (two females and one male) of the MMTV-rtTA transgenic line (3) was received as a kind gift from Frank Costantini (Columbia University, New York, NY). These mice were of a mixed background on C57BL/6. Offspring of these founders were mated to all three lines of the TRE2-FIP200 transgenic mice. The MMTV-rtTA and the TRE2-FIP200 transgenic mice were of different strains with slightly different breeding characteristics and temperament (the MMTV-rtTA being more active while the FVB/N mice are more sedentary). Because of this reason we wanted to know the most advantageous composition of the breeding pairs. For each line (b1, b7, and c3) we set up two mating pairs with opposite gender distribution: in one pair the female was TRE2-FIP200 and the male was MMTV-rtTA and in the other the sexes were reversed. We wanted to know if one type of pair had any advantages over the other in terms of the length of time until the first litter, size of the litter, possible destruction of the litter, and the overall fitness of the litter. At the end we concluded that gender distribution had no influence on the overall reproductive success in these crossings.

d. Detecting both transgenes (TRE2-HA-FIP200-IRES2-EGFP-hβG3' and MMTV-rtTA) in the double-transgenic offspring

The TRE2-HA-FIP200-IRES2-EGFP-hβg3' transgene was detected as previously described. To detect the MMTV-rtTA transgene primers rtTA01, 5'-cattgagatgttagataggc-3', and rtTA02, 5'-aagttgtttttcttaatccgc-3', were used in the following PCR conditions: denaturation at 94 °C for 3 minutes, 32 cycles of amplification (94°C for 50 s, 60°C for 1 min 20 s, and 72°C for 2 min) followed by a 2-min extension at 72 °C (3).

e. Expose the double-transgenic offspring to doxycycline and screening for transgene expression

Litters born to MMTV-rtTA and TRE2-FIP200 parents were genotyped and were fed with 2.0 mg/ml doxycycline and 5% sucrose in their drinking water for 72 hours to detect transgene expression. The drinking water was monitored for consumption and was replaced every day. After the 72 hour treatment period, mice were euthanized and their mammary glands were collected for protein and RNA extraction, and for paraformaldehyde fixation. For RNA and protein extraction, tissues were chilled in liquid N_2 and were subsequently pulverized using mortar and pestle chilled in liquid N_2. Proteins were extracted from the pulverized tissues with Triple-Detergent Buffer (50mM TRIS·Cl, ph=8.0; 150 mM NaCl; 0.02% sodium azide; 0.1% SDS; 100 μg/ml phenylmethylsulfonyl fluoride; 1 μg/ml aprotinin; 1% Nonidet P-40; 0.5% sodium deoxycholate) (6) on ice for 15 minutes and the lysates were cleared by centrifugation at 13,000 RPM in a tabletop microcentrifuge at +4 °C for 15 minutes. The supernatant was used for immunoblotting and immunoprecipitation. RNA was extracted from the pulverized tissues using TRIzol reagent (GIBCO BRL, Carlsbad, California). The process of paraformaldehyde fixation was described earlier.

For immunoblotting lysates were resolved on 7.5% and 10% SDS-PAGE, the proteins were then transferred to nitrocellulose membrane and blotted with rabbit polyclonal anti-FIP200 or anti-hemagglutinin (anti-HA) antibodies (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and with rabbit polyclonal anti-EGFP antibody (Chemicon International, Inc., Temecula, CA). With anti-FIP200 antibody there was no difference of expression between wild-type and transgenic mammary glands under induced conditions. This can be due to the relative paucity of mammary ducts in the mammary fat pad in virgin mice or low levels of transgene expression.

Immunoprecipitation utilized the presence of a covalently linked triple HA tag to the N-terminal of the FIP200 transgenic protein. For immunoprecipitation, the concentration of lysates was adjusted to 1.0 mg/ml with Triple-Detergent Buffer supplemented with protease inhibitors then the lysates were pre-cleared for 1 hour at +4 °C using agarose beads. Then the pre-cleared lysates were incubated with anti-HA agarose beads for 4-16 hours at +4 °C and the immunoprecipitates were washed 5 times (3 minutes each) at +4 °C with Triple-Detergent Buffer supplemented with protease inhibitors. The immunoprecipitates were then resolved on 7.5% SDS-PAGE, the proteins were transferred onto a nitrocellulose membrane and blotted with anti-FIP200 antibody. No transgenic HA-FIP200 was immunoprecipitated in this procedure, which might be due to the fact that the epithelial component is very small in the mammary fat pad of virgin mice. In addition, according to previously published reports, the MMTV-LTR promoter does not direct the expression of the transgene in all the mammary epithelial cells, rather a minority of them show expression and the expression can vary according to the animal's physiological status (7).

Immunohistochemistry using Horse Radish Peroxidase (HRP) streptavidin system with diaminobenzidine (DAB) as the chromogen on paraffin embedded sections was also used to detect transgene expression. The tissues were subjected to heat induced antigen retrieval and then the tissues were blocked with normal goat serum for 1 hour at room temperature. Incubation with the

primary antibodies (anti-HA, anti-EGFP) was performed for 3 hours at 37 °C. After PBS washes (5 times, 5 minutes each) the tissues were incubated with the biotinylated goat-anti-rabbit antibody for 45 minutes at 37 °C and then washed in PBS (5 times 5 minutes each). The incubation with HRP-Streptavidin was carried out at room temperature for 30 minutes. Immunohistochemistry showed moderate to strong staining with anti-HA antibody and weak to moderate staining with anti-EGFP antibody in the mammary epithelium, however there was also moderate background staining in the adipose tissue, endothelial cells, and connective tissue.

Finally, RNA isolated from the mammary glands of double transgenic animals treated with doxycycline was amplified using the Superscript III First Strand Synthesis System (Invitrogen, Carlsbad, California) and then subjected to Polymerase Chain Reaction (PCR) employing appropriate primers. Primers were designed that could selectively amplify only the transgenic FIP200, but not the endogenous FIP200. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a positive control for the reverse transcription step. In one double transgenic line (c3) the message for transgenic FIP200 could be detected in different samples (virgin and non-virgin) under induced conditions. The message for transgenic FIP200 could also be detected in the salivary gland, but not in the spleen. It was an expected result, since the message for the regulator rtTA can also be detected, in addition to the mammary gland, in the salivary gland, but not in the spleen according to the original description of the MMTV-rtTA transgenic line (3). Unlike the message for the transgenic FIP200, the message for GAPDH was detected in all of these organs proving that the reverse transcription step worked. Contrary to the expectations, neither the b1 nor the b7 double transgenic animals showed any expression of the FIP200 transgene under induced conditions.

When transgene expression in the single transgenic c3 animals was assessed with RT-PCR we found surprising results. The message for transgenic FIP200 was not detected in the mammary gland (as expected), but was detected in the salivary gland and spleen. The presence of the message for transgenic FIP200 in the salivary gland and spleen was an unexpected result. One possible explanation for this phenomenon is that there is tissue-restricted, non-specific expression of the transgenic FIP200 in the single transgenic animals of the c3 line. However, no expression of the transgenic FIP200 in our target tissue (mammary gland) makes us optimistic that the c3 double transgenic animals will be suitable for our purpose.

In order to address the difficulties we encountered in our double transgenic mice we plan to perform the following experiments:

1. Pregnancy carried to full term and subsequent lactation increase the expression of any transgene in MMTV-LTR systems, therefore we are planning to use double transgenic animals that have undergone two pregnancies and are in their peak lactation (3-7 days) period. We will assess the transgene expression in these animals with RT-PCR, immunoprecipitation, and immunohistochemistry.
2. It is also possible that the expression of the FIP200 transgene is significantly higher if the animal is homozygous for both transgenes (MMTV-rtTA and TRE2-FIP200). We will generate such doubly homozygous animals.
3. To improve the detection of the transgene in immunohistochemistry we will plan to use frozen sections and other blocking procedures (like the DakoCytomation Protein Block, Serum Free or DakoCytomation Biotin Blocking System) when using paraformaldehyde-fixed paraffin embedded sections. Additionally, we will use other antigen retrieval methods, like digestion with pepsin, pronase, proteinase K, or trypsin.

f. Expansion of both colonies (TRE2-FIP200 and MMTV-rtTA transgenic lines)
The colonies of both the TRE2-FIP200 and the MMTV-rtTA transgenic lines are expanded to accommodate the increased demands for animals in the subsequent experiments.

Aim 2. Effects of the FIP200 transgene on lobulo-alveolar development of the mammary gland during pregnancy, Months 11-18

a. Mating transgenic lines (TRE2-FIP200 and MMTV-rtTA) to create double-transgenic offspring
Two mating pairs of TRE2-FIP200 and MMTV-rtTA for the c3 line and two mating pairs of the TRE2-FIP200 and MMTV-rtTA for the b1 line were set up to generate offspring of different genotype.

b. Mating the double-transgenic offspring with each other
The double transgenic offspring from the same litter were used to mate with each other to generate the target mice in which the development of the mammary gland can be studied in virgin mice with or without doxycycline treatment. Selected double transgenic females from the same litter will be mated with males and the development of their mammary gland during pregnancy with or without doxycycline treatment will be assessed using mammary gland whole mounts and routine paraformaldehyde-fixed, paraffin embedded sections. The mammary gland morphology will be compared to that of wild-type littermates with or without doxycycline treatment.

Training Accomplishments

Classes:
Spring Semester, 2004
Ethical Issues and Professional Responsibilities
Spring Semester, 2005
Mouse Pathology and Transgenesis

References

1. Hogan, B. B., Rosa; Constantini, Frank; Lacy, Elizabeth. 1994. Production of transgenic mice. In *Manipulating the Mouse Embryo*. B. B. Hogan, Rosa; Constantini, Frank; Lacy, Elizabeth, ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, p. 217.
2. 1989. Analysis and Cloning of Eukaryotic Genomic DNA. In *Molecular Cloning - A Laboratory Manual*, Vol. 2. J. Sambrook, Fritsch, E.F., Maniatis, T., ed. Cold Spring Harbor Laboratory Press, Plainview, NY, p. 9.16.
3. Hsu, W., R. Shakya, and F. Costantini. 2001. Impaired mammary gland and lymphoid development caused by inducible expression of Axin in transgenic mice. *J Cell Biol 155:1055.*
4. Mahler, J. F., W. Stokes, P. C. Mann, M. Takaoka, and R. R. Maronpot. 1996. Spontaneous lesions in aging FVB/N mice. *Toxicol Pathol 24:710.*
5. Nieto, A. I., G. Shyamala, J. J. Galvez, G. Thordarson, L. M. Wakefield, and R. D. Cardiff. 2003. Persistent mammary hyperplasia in FVB/N mice. *Comp Med 53:433.*
6. 1989. Detection and Analysis of Proteins Expressed from Cloned Genes. In *Molecular Cloning - A Laboratory Manual*, Vol. 3. J. Sambrook, Fritsch, E.F., Maniatis, T., ed. Cold Spring Harbor Laboratory Press, Plainview, NY, p. 18.32.
7. Choi, Y. W., D. Henrard, I. Lee, and S. R. Ross. 1987. The mouse mammary tumor virus long terminal repeat directs expression in epithelial and lymphoid cells of different tissues in transgenic mice. *J Virol 61:3013.*